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Human bronchial epithelial cells injury and cytokine production induced by *Tityus serrulatus* scorpion venom: An *in vitro* study

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ABSTRACT

Tityus serrulatus is the scorpion specie responsible for the majority of scorpion sting accidents in Brazil. Symptoms of envenomation by *Tityus serrulatus* range from local pain to severe systemic reactions such as cardiac dysfunction and pulmonary edema. Thus, this study has evaluated the participation of bronchial epithelial cells in the pulmonary effects of *Tityus serrulatus* scorpion venom (Tsv). Human bronchial epithelial cell line BEAS-2B were utilized as a model target and were incubated with Tsv (10 or 50 μg/mL) for 1, 3, 6 and 24 h. Effects on cellular response of venom-induce cytotoxicity were examined including cell viability, cell integrity, cell morphology, apoptosis/necrosis as well as cell activation through the release of pro-inflammatory cytokines IL-1β, IL-6 and IL-8. Tsv caused a decrease in cell viability at 10 and 50 μg/mL, which was confirmed by lactate dehydrogenase (LDH) measurement. Flow cytometry analyses revealed necrosis as the main cell death pathway caused by Tsv. Furthermore, Tsv induced the release of IL-1β, IL-6 and IL-8. Altogether, these results demonstrate that Tsv induces cytotoxic effects on bronchial epithelial cells, involving necrosis and release of pro-inflammatory cytokines, suggesting that bronchial epithelial cells may play a role in the pulmonary injury caused by Tsv.

1. Introduction

Scorpion stings are considered a global medical-sanitary problem in many tropical and subtropical countries (Chippaux and Goyffon, 2008). In Brazil, the genus *Tityus* is the main genus responsible for the scorpion accidents in humans with the specie *Tityus serrulatus* causing the majority of severe cases and mortality in southeast and northeast regions (Reckziegel and Pinto Jr, 2014). Pulmonary edema is a frequent finding in patients stung by this scorpion, especially among children, in whom the number of severe cases and the lethality rate are increased (Reckziegel and Pinto Jr, 2014; Bucaretchi et al., 1995; Deshpande and Akella, 2012; Pucca et al., 2015; Amaral et al., 1993). Two distinct mechanisms have been suggested to explain the development of pulmonary edema: acute left ventricular failure resulting from massive catecholamine and inflammatory mediators release (Benvenuti et al., 2002; Freire-Maia et al., 1978), which leads to an increase in vascular permeability, and consequently extravasation of blood plasma in the alveolar space (Deshpande and Akella, 2012). Such inflammatory effect is an important event and a group of researches proposed the use of the term scorpion venom respiratory distress syndrome instead of pulmonary edema induced by scorpionism (D'Suze et al., 1999).

In addition to the pulmonary edema, literature shows that scorpion envenomation induces a systemic inflammatory response characterized by a massive and fast release of cytokines (Magalhães et al., 1999; Fukuhara et al., 2003; Pessini et al., 2003; Fialho et al., 2011). Different cytokines are released following scorpion envenomation both in patients as well as in mice exposed to various species of scorpion venom (Fukuhara et al., 2003; Petricevich, 2010). Indeed, an increase of interleukins (IL)-1α, IL-1β, IL-6, IL-8,

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IL-10, Interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α, were observed in the serum of patients who were stung by *T. serrulatus* (Magalhães et al., 1999; Fukuhara et al., 2003). In addition, it has been demonstrated that the increased levels of IL- 1β, IL-6, IL-8, TNF-α and IL-10 in patients envenomed by *Tityus serrulatus* venom (Tsv) were positively correlated with the severity of envenomation (Fukuhara et al., 2003). Moreover, a study developed by Meki et al. (2003) evaluating scorpion accidents from Egypt, also described a positive correlation between IL-8 release and the severity of envenomation.

Bronchial epithelial cells constitute the main layer of the lung airways and play a wide variety of critical roles in the defense of the lungs against the entry of noxious substances (Rennard et al., 1994). These cells, not only form a passive barrier, but also play an active role in the pulmonary immune response, being able to produce a variety of mediators initiating and perpetuating inflammatory responses inducing the recruitment and activation of lung structural cells and leukocytes (Van der Velden et al., 1999). A number of studies have shown the involvement of immune cells on envenoming caused by Tsv. Petrichevich (2002); Petrichevich et al. (2007; 2008) showed that incubation of these cells with Tsv increased the production of the inflammatory mediators IL-1α, IL-1β, TNF-α, IL-6 and IFN-γ. In addition, an increase in the production of IL-6 and TNF-α by J774.1 macrophages was observed after stimulation with Tsv or its toxins (Ts1, Ts2 and Ts6) (Zoccal et al., 2011). Despite the demonstrated effects of the scorpion venom on the airways and the involvement of cytokines and immune cells in the envenoming caused by Tsv, there is a lack of knowledge regarding the participation of other cells, such as bronchial epithelial cells, in scorpion venom inducing pulmonary injury.

Therefore, the aim of the present work was to study the biological effects of Tsv on human bronchial epithelial cell focusing on cell cytotoxicity, cell death, as well as the ability of the venom to induce cytokines release.

2. Material and methods

2.1. Venom

The lyophilized crude *T. serrulatus* scorpion venom (Tsv) was obtained from the Butantan Institute. The venom was stored at −20 °C until the time of use, when it was diluted in sterile phosphate-buffered saline (PBS).

2.2. Cell culture

The human bronchial epithelial cell line (BEAS-2B) provided by Dr. Roger Chammas (School of Medicine of University of São Paulo, Brazil) was used as the venom target. BEAS-2B cells were maintained at subconfluent levels in growth medium consisting of bronchial epithelial basal medium (BEBM) (Lonza) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic and incubated at 37 °C in a humidified atmosphere containing 5% CO2. The cells were maintained at subconfluence densities and passage every two days. BEAS-2B were plated in 96 well plates (1×10^4 cell/well) and incubated for 48 h for cell adherence. After this period, the cells were incubated with Tsv at concentrations of 10 or 50 μg/ for 1, 3, 6 and 24 h.

2.3. Cell viability assay

Mitochondrial activity was measured to assess bronchial epithelial cell viability, as described previously (Franco et al., 2016). This analysis was based on cell mitochondrial measured by the 3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) based cytotoxicity assay (Sigma Aldrich, St. Louis, MO, USA). After incubation with venom for 1, 3, 6 and 24 h, the cells were washed with 100 μL of PBS and MTT was added to cell culture to a final concentration of 0.5 μg/mL and the cells were incubated for 3 h, at 37 °C. After the incubation time, 100 μL of isopropanol was added to each well to dissolve the formazan crystals. The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 620 nm. Each sample was assayed in triplicate wells, in at least three independent experiments.

2.4. Cell integrity

To confirm the cytotoxicity caused by the venom, the enzymatic activity of Lactate dehydrogenase (LDH) present in the supernatant of cultures was determined as the parameter of cell integrity, as previously described (Villalobos et al., 2007). The effect of Tsv on cell permeability of bronchial epithelial cell wall was evaluated by the release of LDH at 1, 3, 6 and 24 h, in the supernatant of endothelial cell culture. For this purpose, the BEAS-2B cells were incubated with Tsv or BEBM medium only (control), and LDH activity was determined in 20 μL of cell supernatant by the addition of 200 μL of substrate containing 200 mm NaCl, 0.2 mM pyruvate, 1.6 mM NADH, by using a commercial kit (Labtest, Minas Gerais, Brazil). The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 340 nm. The results were expressed by the decrease of the optical density values, resulting from the oxidation of NADH in the presence of pyruvate. Each sample was assayed in triplicate wells, in at least three independent experiments.

2.5. Light microscopy

BEAS-2B cells were photographed before and after 24 h incubation of Tsv (50 μg/mL) for morphology analysis. An TS-100 light microscope (Nikon, objective 40× and 100×) coupled to a video camera (CCD DS-Fi 1, Nikon) and connected to a microcomputer was used for capturing images. BEAS-2B cell counting was performed automatically by the software ImageJ.

2.6. Analysis of apoptosis/necrosis by flow cytometry

The assessment of the apoptotic and necrotic effects induced by Tsv on BEAS-2B was determined by flow cytometry, using a kit containing annexin V-FITC and 7-amino-actinomicyn D (7-AAD) in a FACS Accuri C6 (BD Biosciences, CA, USA). The population of necrotic cells were marked with 7-AAD (7-AAD+), while the cells in apoptosis are marked with annexin-V FITC (AN+) and the population of both necrotic and apoptotic cells is marked as 7-AAD+/AN+ (Nogueira-Pedro et al., 2013).

BEAS-2B cells were plated into 24-well plates (1×10^6 cells/well), followed by incubation for 24 h at 37 °C in a humidified incubator containing 5% CO_2. After this period, the cells were treated with 50 μL of PBS (negative control) or 50 μL of Tsv samples at concentration of 50 μg/mL. After 24 h of treatment, cells were transferred to flow cytometry tubes, centrifuged at 900 *g* for 5 min, the supernatant was discarded and cellular pellet ressuspended in 1 mL of PBS. The same process was repeated, this time with addition of 50 μL of annexin buffer, 3 μL of annexin V-FITC (Becton Dickinson - USA) and 5 μg/mL of 7-amino-actinomicyn D (7-AAD) (Invitrogen - USA) for 20 min protected from light. After incubation, cells were washed and then ressuspended with 200 μL of annexin buffer for data acquisition on flow cytometer. A total of 50.000 events were acquired on FACs Accuri C6 (Becton Dickinson – USA). Cell Quest Pro Analysis (version 5.1 - Becton Dickinson – USA) and

FlowJo Softwares (version 7.4.6 – Tree Star – USA) were used for data acquisition and data analysis respectively. All experiments were performed in triplicates.

2.7. Evaluation of the cytokines IL-1β, IL-6 and Il-8

Supernatants of BEAS-2B cells were used for determination of IL-1β, IL-6 and IL-8 levels, which was quantified using the enzyme-linked immunosorbent assay, as per the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA.) Briefly, 96-well plates were coated with 50 mL of the first capture monoclonal antibody anti-IL-1 β (2 μg/mL), anti-IL-6 (500 pg/mL) and anti-IL-8 (1 μg/mL) and incubated for 18 h, at 37 °C. Following this period, 200 mL of blocking buffer, containing 5% bovine serum albumin in PBS/Tween 20 (Sigma, St. Louis, MO, USA) were added to the wells and the plates were incubated overnight at 4 °C. After washing, 100 μL of either samples or standards were dispensed into each well and the plates incubated for 2 h at 37 °C. Wells were washed, and bounded IL-1β, IL-6 or IL-8 was detected by the addition of the biotinylated monoclonal antibodies anti-IL-1β, anti-IL-6 or anti- IL-8 (5 μg/mL, 50 μL/well). After washing the plates, the volume of 100 μL streptavidin-peroxidase was added and left for 1 h at room temperature (22 °C) followed by further washes. The reading was performed in a multireader Spectra I3 (Molecular Devices Corporation, Sunnyvale, CA, USA) at a wavelength of 450 nm with correction at 570 nm. The sample concentrations were calculated from standard curves obtained with recombinant cytokines.

2.8. Statistical analysis

The statistical significance among the control and venom-treated groups was determined by one-way analysis of variance (ANOVA) followed by the Tukey test. A value of $P < 0.05$ indicated statistical significance.

3. Results

3.1. 1. Effect of Tsv on cell viability

When BEAS-2B cell line was incubated with Tsv (10 and 50 μg/mL) for 1 h, no cytotoxicity was observed (Fig 1). Exposure of BEAS-2B cells to Tsv for 3, 6 and 24 h significantly reduced cell viability at both concentrations studied (10 and 50 μg/mL) when compared to control group (Fig. 1). There was no statistical difference in cell viability between the studied concentrations at 3 h and 6 h period of venom incubation (Fig. 1). However, at 24 h the concentrations of 50 μg/mL caused a statistically significant reduction in cell viability compared with the concentration of 10 μg/mL (Fig. 1).

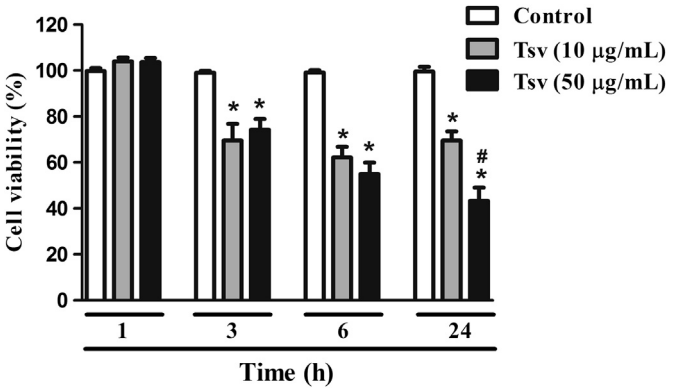

Fig. 1. Effect of Tsv on the viability of BEAS-2B cells. BEAS-2B cells were plated into 96 well plates and incubated for 24 h for cellular adhesion. After this period the venom was added (10 or 50 μg/mL) or cells received only medium (control) and were incubated for 1, 3, 6, or 24 h. Cell viability was determined by MTT assay. Each value represents the mean ± SEM of three independent experiments. *$p < 0.05$ compared to control and #$p < 0.05$ compared to venom 10 μg/mL.

3.2. Effect of Tsv on cell integrity

The enzymatic activity of LDH, present in the supernatant of cell cultures, was taken as the parameter of cell integrity. Cells treated with Tsv at a concentration of 50 μg/mL caused the release of LDH that was significantly greater than the baseline control by 40 and 123% at 6 and 24 h, respectively (Fig. 2). The concentration of 10 μg/mL of Tsv caused an increase of LDH in the supernatant 24 h after venom incubation, that was 26% higher than the group that received only medium (control) (Fig. 2).

3.3. Effect of Tsv on cell morphology

BEAS-2B cells were observed under optical microscope before and after 24 h of Tsv (50 μg/mL) incubation. Tsv caused a significant reduction of 52% in the number of BEAS-2B cells in comparison with control cell (Fig. 3). Furthermore, there is a reduction in cytoplasmic volume in cells that were incubated with Tsv (arrow) (Fig. 3B).

3.4. Analysis of the necrotic/apoptotic effects of Tsv

We next analyzed the effect of Tsv by flow cytometry on apoptosis and necrosis of BEAS-2B cells. Treatment of BEAS-2B cells with Tsv at the concentrations of 50 μg/mL induced apoptosis/necrosis ($AN^+/7AAD^+$) in 43,6% of cell population, necrosis ($7AAD^+$) in 16,4% of cells, and apoptosis (AN^+) in 4,7% of cell (Fig. 4), indicating that necrosis was the main mechanism of cell death induced by Tsv.

3.5. Effect Tsv on cytokine release

To investigate the effect of Tsv on the BEAS-2B activation, the

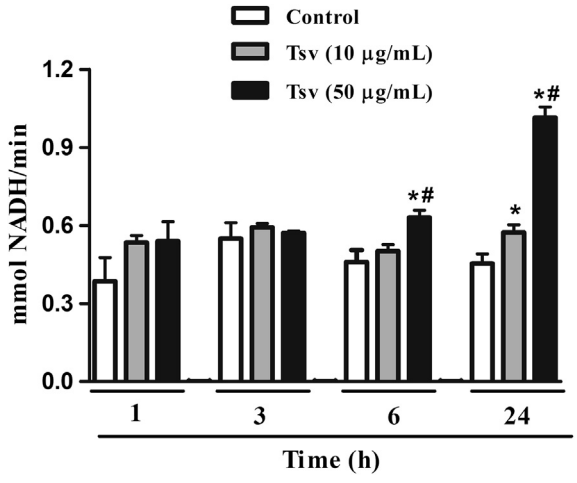

Fig. 2. Effect of Tsv on integrity of BEAS-2B cells. BEAS-2B cells were plated into 96 well plates and incubated for 24 h for cellular adhesion. After this period the venom was added (10 or 50 μg/mL) or cells received only medium (control) and were incubated for 1, 3, 6 and 24 h. Determination of LDH activity was determined by the decrease of the optical density values resulting from the oxidation of NADH in the presence of pyruvate. Each value represents the mean ± SEM of three independent experiments. *$p < 0.05$ compared to control and #p < 0.05 compared to venom 10 μg/mL.

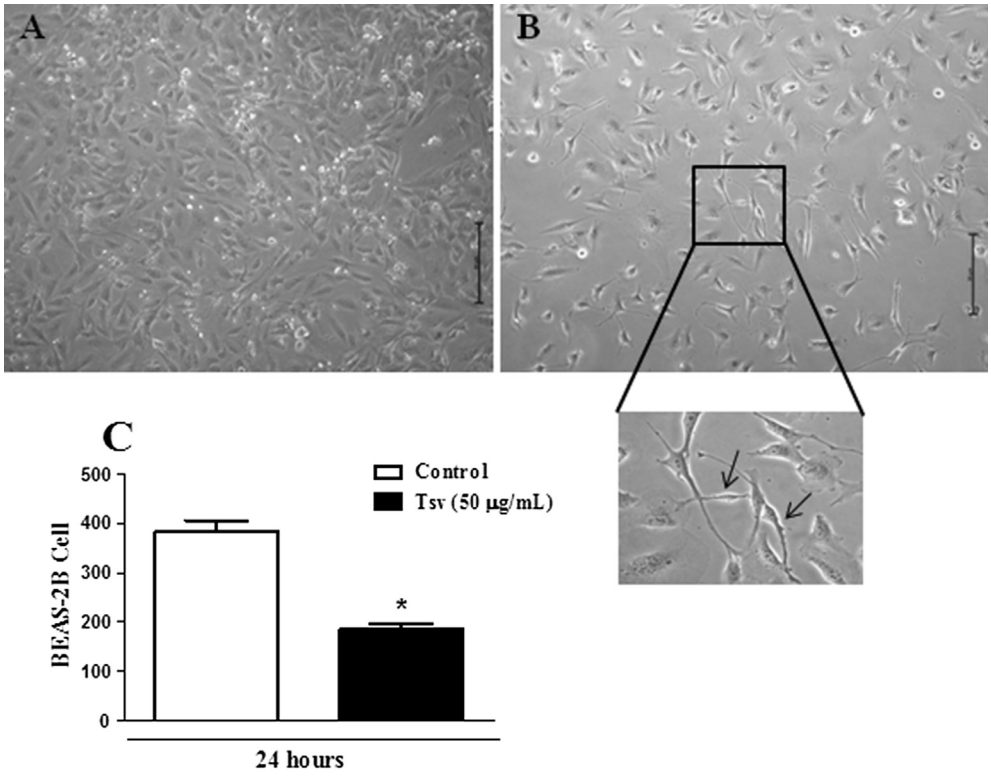

Fig. 3. Morphological alterations caused by Tsv on BEAS-2B cells. (**A**) Control cells. (**B**) Effect of Tsv (50 µg/mL) after 24 h. (**C**) Bar graph showing the cell counting of three independent experiment. Note that Tsv cause a reduction in the number of BEAS-2B cells (B and C) as well as a reduction in the cytoplasm volume (arrow). Magnification 100×, scale bar 50 µm $^{*}p < 0.05$.

measurements of the release of pro-inflammatory cytokines IL-1β, IL-6 and IL-8 after stimulation with Tsv (10 or 50 µg/mL) was done in the cell culture supernatants. As illustrated in Fig. 5A, cells that were incubated with Tsv showed increased levels of IL-1β in the supernatant at 6 h of venom incubation by either 10 or 50 µg/mL. Tsv induced a marked elevation of IL-6 levels from 1 up to 24 h after venom incubation by both concentrations studied (Fig. 5B). Furthermore, the venom significantly induced a marked increment of IL-8 levels at all intervals time analyzed, with maximal increased levels observed at 3 h after Tsv incubation by either 10 or 50 µg/mL (Fig. 5C).

4. Discussion

In the present study, it was demonstrated for the first time that bronchial epithelial cells may be activated by Tsv and play a role in the pulmonary effects of Tsv. For this purpose, the cell line BEAS-2B, which has been extensively used as a model of normal human bronchial epithelial cells (Vieira et al., 2011; Sobkowicz et al., 2014; Baudiβ et al., 2015) was used. These cells functionally respond similarly to bronchial epithelial cells *in vivo* and actively respond to stimulus and treatments (Vieira et al., 2011; Sobkowicz et al., 2014; Baudiβ et al., 2015). In addition, they are frequently used for studies of pulmonary injury and toxicology (Bhowmick and Gappa-Fahlenkamp, 2016).

Here, we used a concentration of 10 and 50 µg of Tsv to evaluate the cytotoxicity on BEAS-2B cells. There is no available data in relation to a concentration of Tsv that could be achieved in human's lung. However, Revelo et al. (1996) demonstrated experimentally, that Tsv (10 µg) could be detected in higher levels in the lung from 30 min to 8 h of a subcutaneous injection into mice. Our study has shown that Tsv causes a decrease in the viability of BEAS-2B cells. Moreover, the fact that Tsv increased LDH release demonstrates that venom leads to cell membrane permeability and suggests a direct effect on bronchial epithelial cell membrane. Although tissue injury with LDH extravasation is expected in accidents induced by *Buthidae* scorpions, as demonstrated in experimental (Correa et al., 1997; Zare-Mirakabbadi et al., 2007; Adi-Bessalem et al., 2008; Chaubey, 2010) and clinical studies (Meki et al., 2003; Aboumaâd et al., 2014) we showed, by the first time, that human bronchial epithelial cells can be affected by the scorpion venom, leading to a direct cell damage.

To further analyze the mechanism by which Tsv acts on BEAS-2B cells, the apoptosis or necrosis was assessed by flow cytometry. Our results showed that necrosis is the predominant cell death mechanism induced by Tsv on these cells. Necrosis induced by scorpion venom was also reported in a dose-dependent manner in different cells, as human neutrophils (Borges et al., 2011) and mouse brain tumor cells (BC3H1) (Caliskan et al., 2013). Necrosis is an important pathway that leads to innate immune-mediated acute inflammation (Kono et al., 2014), it is possible that the necrosis observed in the bronchial epithelial cells induced by Tsv in the present study, could generate inflammation, since the pulmonary pathophysiological effects observed in scorpionism are normally due to cardiogenic and inflammatory events (Amaral et al., 1993; Amaral and Rezende, 1997).

Literature describes that patients envenomed by Tsv present increased levels of pro-inflammatory cytokines in the blood (Magalhães et al., 1999; Fukuhara et al., 2003). However it is not clear which are the cells responsible for the release of these mediators. Inflammatory cells are the mainly source of such mediators, but we cannot exclude the participation of other cells in the tissues affected by Tsv. In the airways, at least macrophages and mast cells can trigger an inflammatory reaction, releasing several chemical

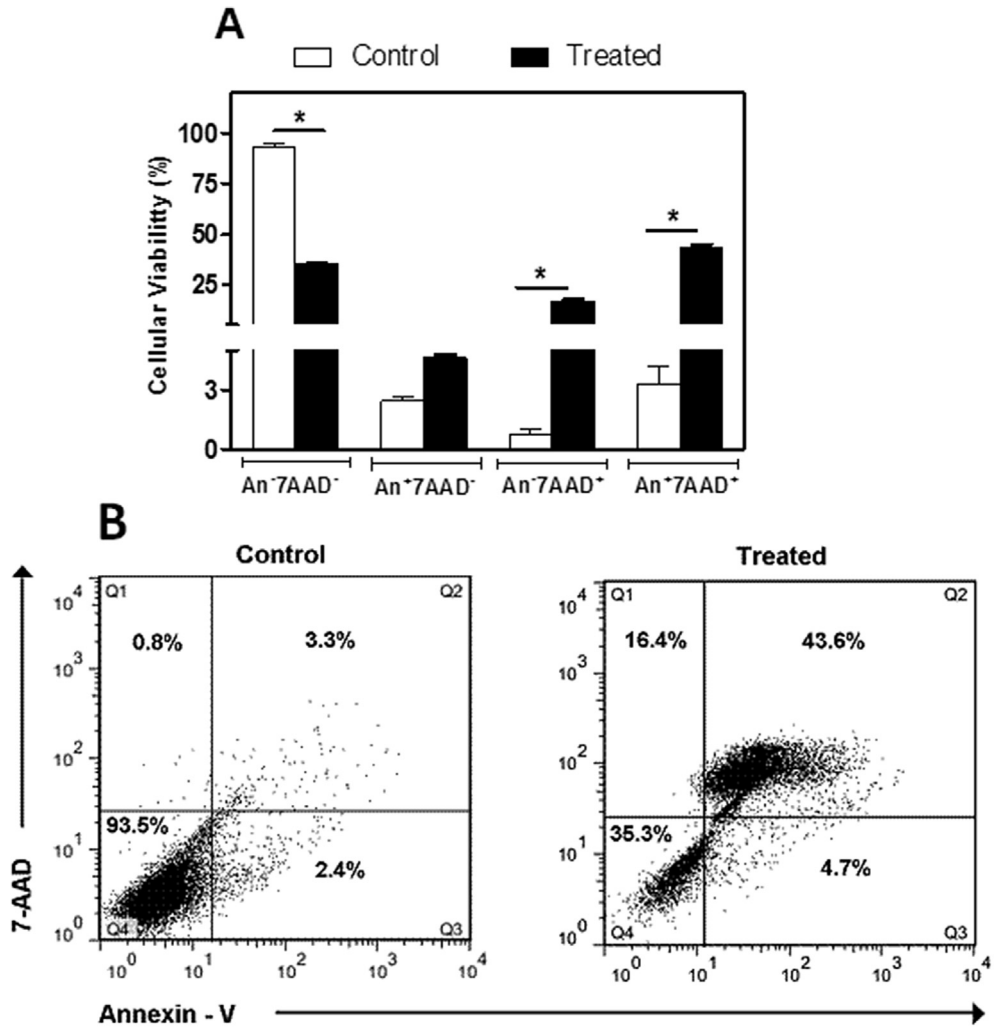

Fig. 4. Analysis of apoptosis/necrosis by flow cytometry. BEAS-2B cells were treated with 50 μg/mL for 24 h, and stained with Annexin V-FITC/7AAD solutions according to the manufacturer's manual, and detected using flow cytometry. (**A**) Column bar graph of apoptotic cells. (**B**) Representative dot plots of Annexin-V/7AAD staining. Annexin $V^-/7AAD^-$ (lower left) cells were represented survivals, Annexin $V^+/7AAD^-$ (lower right) cells were defined as early apoptotic cells, Annexin $V^+/7AAD^+$ (upper right) cells were recognized as apoptotic/necrotic cells, Annexin $V^+/7AAD^-$ (upper left) cells were considered as necrotic cells.

mediators. Concerning the Tsv effect on both cells, it has been shown that the venom is able to activate macrophages to release the pro-inflammatory cytokines IL-6, TNF-α and IFN-γ (Petricevich, 2002; Zoccal et al., 2011), but mast cells do not appear to have a role in the initial phase of airway inflammation in rats induced by Tsv (Zuliani et al., 2013).

As previously described, different cytokines have been found in human scorpionism. However, the involvement of cytokines in human pulmonary inflammation is unknown. IL-8 is a cytokine that is highly active as neutrophil chemokine and it was associated with severe scorpionism and fatal outcome (Meki et al., 2003). It is possible that production of IL-8 by airways epithelial cells could promote pulmonary influx of neutrophils leading to tissue injury. It has been demonstrated that *Tityus zulianus* crude venom is able to induce human neutrophil activation (Borges et al., 2011) and we can speculate that since these cells have reached the lung environment they could mediate lung tissue injury. Recently, Tsv was described as an inflammasome activator leading to IL-1β production by mice macrophages, which present pivotal role in the pathophysiological changes, in the lung injury, such as increased protein content in bronchoalveolar lavage and neutrophil infiltration (Zoccal et al., 2016).

In addition, IL-6 a cytokine also detected after Tsv stimulation is a cytokine with multiple actions that has been detected in humans stung by scorpions as well as in experimental Tsv scorpionism (Pessini et al., 2003). Interestingly, Schütte et al. (1996) found elevated levels of IL-6 and IL-8 in blood and in bronchoalveolar lavage of patients with acute respiratory distress syndrome together with increased numbers of infiltrating neutrophils. Our results showing increased release of IL-1β, IL-6 and IL-8 production by the human bronchial epithelial cells suggests that structural lung cells, in this case bronchial epithelial cells have an important involvement in scorpionism inducing acute respiratory injury. Therefore, a new concept about the capacity of the scorpion venom to stimulate pulmonary inflammation through activation of bronchial epithelial cells was demonstrated in this study. Thus, is plausible that bronchial epithelial cells may play a role in pulmonary inflammation induced by scorpion venom. The suggestion raised here is consistent with the proposed role of different lung epithelial cells in the pathogenesis of acute lung injury/adult respiratory distress syndrome (Gropper and Wiener-Kronish, 2008).

In conclusion, Tsv induces bronchial epithelial cells activation and also cell death through apoptosis and necrosis, suggesting its participation in the acute lung injury induced by Tsv.

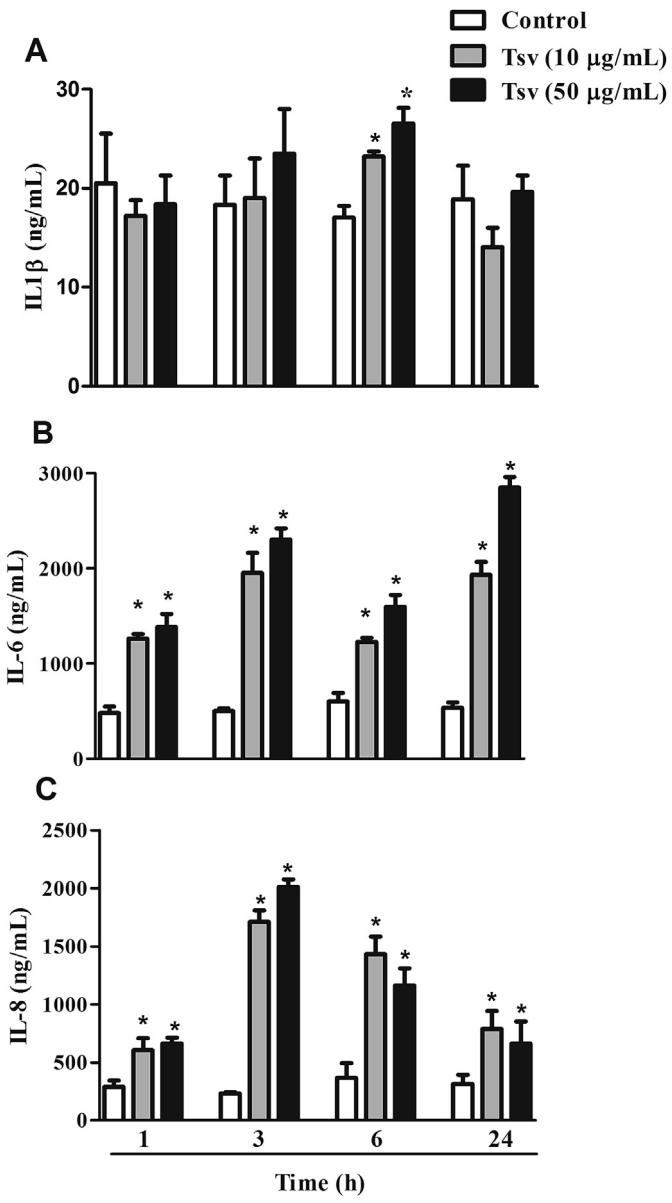

Fig. 5. Effect of Tsv on the release of IL-1β, IL-6 and IL-8 by BEAS-2B cells. BEAS-2B cells were plated in 96-well plates and incubated for 24 h for cell attachment. After this period, Tsv (10 or 50 μg/mL) was added to the cell culture and were incubated for 1, 3, 6 or 24 h. The concentrations of IL-1β, IL-6 and IL-8 were measured by ELISA in the supernatant of cells. *$p < 0.05$ vs. control group.

Conflict of interest

The authors declare that there are no conflicts of interest.

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